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Original Article

Lack of Efect of Short-term Lupin Grain Feeding on Ovulation Rate in Non-prolific Polish Mountain Ewes during the Breeding Season: Ultrasonographic and Endoscopic Assessment of Ovarian Activity

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Abstract

The specific changes in antral follicle numbers and wave-like development have remained unrevealed in cyclic ewes fed high-protein, high-energy lupin grain for 6 days during the luteal phase of the estrous cycle (i.e., short-term nutritional flushing). This study was mainly conducted to determine ovarian effects of the 6-day lupin grain feeding in non-prolific Polish Mountain ewes, using transrectal ovarian ultrasonography and abdominal videoendoscopy. Estrus and ovulations were synchronized in 24 ewes with progestin-releasing intravaginal sponges for 12 days during the middle portion of the breeding season (September-October; 50.0458°N, 19.8406°E). Twenty-four ewes were assigned to three equal groups (n=8 each), including the Control group being fed the maintenance diet (i.e., hay-only), Treatment 1 receiving 500 g of lupin grain once a day, and Treatment 2 receiving 250 g of lupin grain twice a day, from days 9-14 of the synchronized estrous cycle (day 0=first ovulation of the interovulatory period studied). No differences were observed in the mean ovulation rate among the three groups of Polish Mountain ewes ($P>0.05$). Ovarian antral follicles emerging in the penultimate wave of the estrous cycle in Treatment 2 ewes had a longer growth phase ($P<0.05$) and attained a greater diameter ($P<0.05$) before ovulation, in comparison to those in the other two groups. A final wave of the interovulatory interval emerged ~1 day earlier in Treatment 2 than in Treatment 1 ewes ($P<0.05$). Nutritional supplementation with lupin grain increased the number of 3-mm follicles in Treatment 2 ewes ($P<0.05$). The results of this study indicated that short-term nutritional flushing with lupin grain from mid- to late luteal phase did not consistently enhance ovulatory responses in non-prolific genotypes of ewes. Although the administration of lupins altered the timing of wave emergence, ovulatory follicle diameter, or duration of different stages of the follicular lifespan, it failed to increase the number of ovulatory follicles emerging in the penultimate and final waves of the estrous cycle in non-prolific Polish Mountain sheep.

Keywords: Sheep, Ovulation rate, Lupin grain, Flushing, Ultrasonography

Absence d'effet de l'alimentation au Grain de Lupin à Court Terme Sur le Taux D'ovulation des Brebis de Montagne Polonaises Non Prolifiques Pendant la Saison de Reproduction: Évaluation Échographique et Endoscopique de l'activité Ovarienne

Résumé: Les changements spécifiques dans le nombre de follicules antraux et le modèle de développement en

forme de vague chez les brebis cycliques recevant des grains de lupin à haute teneur en protéines et à haute énergie pendant 6 jours pendant la phase lutéale du cycle œstral (bouffées nutritionnelles à court terme) restaient inconnus. Le principal objectif de cette expérience était d'utiliser l'échographie et la vidéoendoscopie ovariennes transrectales pour déterminer les effets ovariens d'une alimentation de 6 jours au grain de lupin chez les brebis de montagne polonaises non prolifiques. Les œstrus et les ovulations de 24 brebis ont été synchronisés par l'insertion pendant 12 jours d'éponges intravaginales à libération de progestatif au milieu de la saison de reproduction (septembre-octobre; 50,0458° N, 19,8406° E). Huit brebis ont reçu le régime témoin (foin uniquement), tandis que les groupes nourris au lupin (chacun n = 8) ont reçu 500 g de lupin une fois par jour (traitement 1) ou 250 g de lupin deux fois par jour (Traitement 2), du 9e au 14e jour du cycle synchronisé (jour 0 = première ovulation de la période interovulatoire étudiée). Il n'y avait aucune différence (P>0,05) dans le taux d'ovulation moyen entre les trois groupes de brebis de montagne polonaises étudiées. Les follicules ovariens antraux émergeant dans l'avant-dernière vague du cycle œstral du traitement 2, les brebis ont eu une phase de croissance plus longue (P<0,05) et ont atteint un diamètre supérieur (P<0,05) avant l'ovulation par rapport aux deux autres groupes d'animaux. Une dernière vague de l'intervalle interovulatoire est apparue ~1 jour plus tôt chez les brebis soumises au traitement 2 comparé au traitement 1 (P <0,05). La supplémentation nutritionnelle avec des grains de lupin a augmenté (P<0,05) le nombre de follicules de 3 mm dans les brebis du traitement 2. Les résultats actuels indiquent qu'une complémentation nutritionnelle à court terme à base de grain de lupin durant la phase lutéale moyenne ainsi qu'à la fin de la phase lutéale n'améliore pas systématiquement les réponses ovulatoires dans les génotypes non prolifiques de brebis. Même si l'administration de lupins a modifié le moment de l'émergence des vagues, le diamètre du follicule ovulatoire ou la durée des différentes étapes de la vie folliculaire, elle n'a pas réussi à augmenter le nombre de follicules ovulatoires émergeant dans l'avant-dernière et dernière vague du cycle œstral chez les moutons de montagne polonais non prolifiques.

Mots-clés: Mouton; Taux d'ovulation; Grain de lupin; Rinçage; Echographie

Introduction

Nutrition is one of the most important extrinsic factors influencing fertility in small ruminants (Scaramuzzi et al., 2006; Arellano-Rodriguez et al., 2007; Scaramuzzi et al., 2011; Meza-Herrera and Tena-Sempere, 2012). More than one effect of nutrition on the ovulation rate in sheep have been recognized to date including: the static effect, the dynamic effect, and the intermediate effect (Viñoles, 2003). The static effect refers to the generally higher ovulation rate in heavyweight ewes compared with lighter animals. The dynamic effect is defined as a rise in the ovulation rate due to an increase in live weight and body condition score shortly before the estrous period and mating. The intermediate effect is referred to as the short-term supplementation with a high-energy, high-protein diet resulting in a higher ovulation rate without any changes in live weight or body condition score (Viñoles, 2003).

Lupins are used as dietary supplements (e.g., *Lupinus angustifolius*: 13 MJ ME/kg DM and 320 g CP/kg DM) (Kendall et al., 2003). It has been reported that short-term lupin feeding (i.e., 4-6 days) during the luteal phase of the estrous cycle increases the mean ovulation rate in cycling ewes (Scaramuzzi et al., 2006; Scaramuzzi et al., 2011). Results of many studies have shown a significant increase in the mean ovulation rate of ewes fed lupins during various periods before breeding (Hill, 1988; Scaramuzzi et al., 2011). However, the results of different studies are not uniform in showing marked responses (Hill, 1988). Physiological processes responsible for the intermediate effects are not completely understood nor is the precise ovarian mechanism by which nutritional flushing with lupins increases the ovulation rate in sheep.

One possibility can be the metabolic effect of short-term nutritional flushing, and the increase of ovulation rate in response to the energy and/or protein content of the lupin grain (Scaramuzzi et al., 2006). Alternatively, it has been suggested that lupin ingredients may directly modulate folliculogenesis in ewes by increasing ovarian sensitivity to gonadotropic hormones (i.e., hormonal effect) (Kendall et al., 2003). Cultivated varieties of grain legumes have

been identified as excellent sources of phytoestrogens enhancing ovarian responsiveness to luteinizing hormone (Petterson and Fairbrother, 1994; Jayasena et al., 2004). Ovarian antral follicles in sheep grow in the wave-like pattern throughout the 17-day interovulatory interval (Bartlewski et al., 2011). In cyclic ewes, one to four small antral follicles (2-3 mm in diameter) emerge approximately every 4 days to attain an ostensibly ovulatory size of ≥ 5 mm in diameter (Bartlewski et al., 2011). In prolific breeds of sheep, ovarian follicles from the last two waves of the estrous cycle can ovulate to give a higher ovulation rate This rate is lower in non-prolific genotypes, in which only follicles from a final wave of the cycle ovulate and a proportion of potential ovulatory follicles regress before the estrous phase. Comparing the two types of breeds, it has been reported that nearly 100% of such follicles ovulate in prolific ewes (Bartlewski et al., 2011). Therefore, there exist at least three possible mechanisms whereby lupin grain supplement exerts its effects on ovulation rate in non-prolific strains of sheep, including 1) an addition of antral follicles from the penultimate wave to the ovulatory follicle cohort, 2) an increase in the number/percentage of ovulatory follicles emerging in a final wave, or 3) both changes in antral follicle kinetics.

This study was carried out to examine the effects of short-term nutritional flushing with lupin grain on ovarian activity and ovulation rates in non-prolific Polish Mountain sheep. The purpose of this experiment was to employ ultrasonographic imaging and videoendoscopy of ovaries to document antral follicle development, ovulation rate, and corpus luteum formation in cyclic Polish Mountain ewes receiving lupins from days 9 to14 after ovulation.

Material and Methods

Animals and experimental procedures. All procedures followed the standards of European Union directives for Animal Experiments and were conducted under local animal care/bioethics committee authorization. The present experimental design was based largely on a study performed by Somchit et al. (2007). The statistical population of the present study consisted of 24 clinically healthy, multiparous, non-prolific Polish Mountain ewes aged 2-3 years and with the mean body weight of 62.5±4.1 kg. These animals were entered into the study from the field research station of the Agricultural University of Kraków, Poland, situated in Bielany (50.0458°N, 19.8406°E). The study was conducted in September, amid the regular breeding season, and the animals were kept in separate groups in a barn with ambient light and temperature. Estrus and ovulations were synchronized with progestin-releasing intravaginal sponges (Chronogest®, 45 mg of flugestone acetate; Intervet International B.V., Boxmeer, Holland) inserted for 12 days. Estrus was detected twice daily (2x20 min), starting from the 2nd day after sponge withdrawal, with two fertile rams being fitted with an apron preventing penetration during mounting. Ovulations were defined as a disappearance of the largest visible antral follicles followed by a formation of corpora hemorrhagica approximately 24 h after follicle rupture (Duggavathi et al., 2003b).Prior to the present experiment, the ewes' diet consisted of formulated concentrate to provide 100% of nutritional requirements (Norms, 1993). In addition, all animals had free access to good quality alfalfa hay (crude protein: 8.2% of dry matter, total extractable lipids: 2.9% of dry matter, and moisture: 15.0%), water, and mineralized salt licks containing herbal extracts with anti-parasitic properties (Star Bloc Phyto Vers, Guyokrma Ltd., Prague, the Czech Republic) (Nosal et al., 2016). The ewes were divided by weight into three groups, namely a control group (n=8) and two lupin-fed groups (Treatment 1 and Treatment 2, each n=8). The ewes in the lupin-fed groups received the maintenance diet from the day of sponge insertion until day 9 (day 0=first ovulation of the interovulatory interval). On the 9th day, the ewes in these groups were switched to a lupin grain diet (*Lupinus angustifolius*: 500 g/ewe/day; Treatment 1; 500 g at 8:00 a.m.; Treatment 2: 250 g at 8:00 a.m. and

250 g at 6:00 p.m.) until day 14 (a total of 6 days). The animals in the Control group received the hay-only diet throughout the study period. The underlying reason for testing two treatment regimens (i.e., lupin grain feeding intervals) lies in the fact that *in situ* nitrogen disappearance rate for raw lupins is ~83% after 12-h incubation in the rumen (Kung et al., 1991). Based on the rapid ruminal absorption and lupin grain utilization, two groups were designed to evaluate and compare the "intermittent" (Treatment 1) and more "stable" (Treatment 2) influence of its constituents. The animals in the Treatment 1 or Treatment 2 group were trained to eat the lupin grain (crude protein: 48.3% of dry matter, total extractable lipids: 5.0% of dry matter, and moisture: 11.8%) over 1-2 days before the beginning of the experiment.

Monitoring ovarian function. Transrectal ovarian ultrasonography was performed every morning from day 8 of the estrous cycle studied until ovulation using the Aloka ProSound 2 scanner (Hitachi Aloka Medical, Tokyo, Japan) equipped with a stiffened (plastic extension) 7.5-MHz linear array transducer. Digital ultrasonograms of both ovaries (DICOM) at a resolution of 640x480 pixels were recorded directly on a computer connected to an ultrasound diagnostic system. Laparoscopy was performed approximately 7 days after the second ovulation of the estrous cycle studied to enumerate corpora lutea. Each ewe received a single intramuscular injection of 0.4 ml of Nefrasin vet. (containing 20 mg/ml of xylazine hydrochloride; aniMedica Polska, Gdynia, Poland) and local subcutaneous injections of 1.5-2 ml of Polocainum Hydrochloricum 2% Cum Adrenalin 0,005% (Biowet, Drwalew, Poland). The laparoscopic procedure was performed with the TELE PACK VET X LED video endoscope (KARL STORZ GmbH & Co. KG, Tuttlingen, Germany) and all recordings were saved onto a USB drive.

Follicle classification, follicular wave kinetics and statistical analyses. Follicles were grouped into four size classes: 2-mm, 3-mm, 4-mm, and ≥ 5-mm follicles (Duggavathi et al., 2003a). Follicular waves were defined as the synchronous growth of a group of follicles from 2-3 to ≥5 mm in diameter before regression or ovulation; follicles emerging within 24 h were included in a wave (Baby and Bartlewski, 2011a; Baby and Bartlewski, 2011b). In this study, the following variables were noted: the day of wave emergence, number of all follicles, number and proportion of ovulatory follicles in the penultimate, and the last wave of the estrous cycle studied. All antral follicles of the waves were analyzed for their maximum diameter, duration of the growth phase, static phase, and regressing phase, and the mean growth rate (Bartlewski et al., 1999). The growth phase of the follicular lifespan was defined as the time taken to grow from 2-3 mm to the maximum diameter. The regressing phase referred to the number of days taken by a follicle to regress from its maximum diameter to 2-3 mm. The time between the end of growth and the onset of regression or ovulation was the static phase of the follicular lifespan. The number and diameter of all ovulating follicles of waves were also recorded.

The collected data were analyzed using SigmaPlot ® software (version 11.0, 2010 Systat Software, Inc. Richmond, CA, USA). The results were presented as means±standard error of mean, unless otherwise indicated. Based on the findings, the p-value <0.05 was considered significant. All data sets were initially tested for outliers using the Dixon Q test. All single-time responses and follicular wave characteristics were analyzed using a one-way analysis of variance (ANOVA). The analysis of changes in follicle populations (based on daily follicle counts) was carried out using two-way repeated measures ANOVA, with time (i.e., day) as the within-subjects factor, sheep as the repeated measure, and group (Control, Treatment 1, or Treatment 2) as the between-subjects factor. The data with non-homogeneous variances were transformed by $\log_n$, and if still not normalized, they were analyzed using a non-parametric equivalent of two sample t-test (i.e., the Mann-Whitney U test).

Results

No significant differences were observed among the lupin-fed and Control groups of ewes regarding the mean duration of the interovulatory period, ovulation rate, and numbers of detected luteal structures (Table 1; P>0.05). Ovarian antral follicles in the penultimate wave of the estrous cycle had a longer growing phase in Treatment 2 group by approximately 2 days (P<0.05), compared with both the Control and Treatment 1 groups. The duration of the regressing phase of follicle lifespan was greater in the Control compared with Treatment 1 ewes (P<0.05). The diameter attained by ovulating follicles in this wave was greater (P<0.05) in Treatment 2 group than that in the other two groups. The emergence of the final wave of the interovulatory interval occurred ~1 day earlier (P<0.05) in Treatment 2, compared with Treatment 1 ewes. No other differences were noticed among the three groups for any characteristics of the last two follicular waves of the estrous cycle.

Table 1. Comparisons of ovulation rates/numbers of detected luteal structures, estrous cycle duration, and characteristics of follicular waves (penultimate and final wave of the estrous cycle studied; means±SEMs) among Control (hay-only diet) and lupin-fed groups

Variables	**Control** (n=8)	**Treatment 1** (n=8)	**Treatment 2** (n=8)
Ovulation rate	1.6±0.2	1.7±0.2	1.6±0.2
Number of luteal structures at mid-cycle	1.5±0.2	1.7±0.2	1.5±0.2
Interovulatory interval (days)	16.9±0.2	16.7±0.4	16.8±0.3
Penultimate wave			
Day of emergence*	9.2±0.2	9.4±0.4	9.4±0.7
Number of follicles	2.1±0.4	2.7±0.4	1.7±0.3
Number of ovulatory follicles	0.12±0.12	0.25±0.25	0.29±0.18
Percentage of ovulating follicles	6.2±6.2	8.3±8.3	21.4±14.9
Growth phase (days)	2.0±0.5^{a}	1.8±0.4^{a}	3.8±0.5^{b}
Growth rate (mm/day)	1.6±0.2	1.7±0.1	1.1±0.2
Static phase (days)	2.3±0.6	2.2±0.4	2.2±0.7
Regressing phase (days)	2.8±0.4^{a}	1.4±0.1^{b}	1.9±0.4^{ab}
Regression rate (mm/day)	1.0±0.1	1.7±0.2	1.2±0.2
Maximum follicle diameter (mm)	6.2±0.6	5.9±0.9	6.5±0.3
Ovulatory follicle diameter (mm)	5.5±0.2^{a}	5.2±0.2^{a}	6.2±0.3^{b}
Final wave			
Day of emergence*	12.4±0.5^{ab}	12.8±0.4^{a}	11.7±0.4^{b}
Number of follicles	2.2±0.5	2.9±0.5	2.6±0.3
Number of ovulatory follicles	1.5±0.2	1.5±0.3	1.4±0.3
Percentage of ovulating follicles	80.0±9.8	58.3±14.1	52.1±9.7
Growth phase (days)	2.1±0.4	1.8±0.3	2.2±0.3
Growth rate (mm/day)	1.3±0.1	1.6±0.1	1.4±0.1
Static phase (days)	2.7±0.5	2.3±0.4	2.0±0.2
Maximum follicle diameter (mm)	5.9±0.3	5.5±0.2	6.1±0.1
Ovulatory follicle diameter (mm)	6.1±0.3	4.9±0.7	5.9±0.9
Interwave interval (days)	4.2±0.4	4.4±0.2	3.4±0.4

Treatment 1: 500 g of lupin grain at 8:00 a.m. and Treatment 2: 250 g of lupin grain at 8:00 a.m. and 250 g at 6:00 p.m. from days 9 to 14

*Day 0=first ovulation of the interovulatory interval studied

Within rows, different letters denote significant differences (P<0.05) among groups.

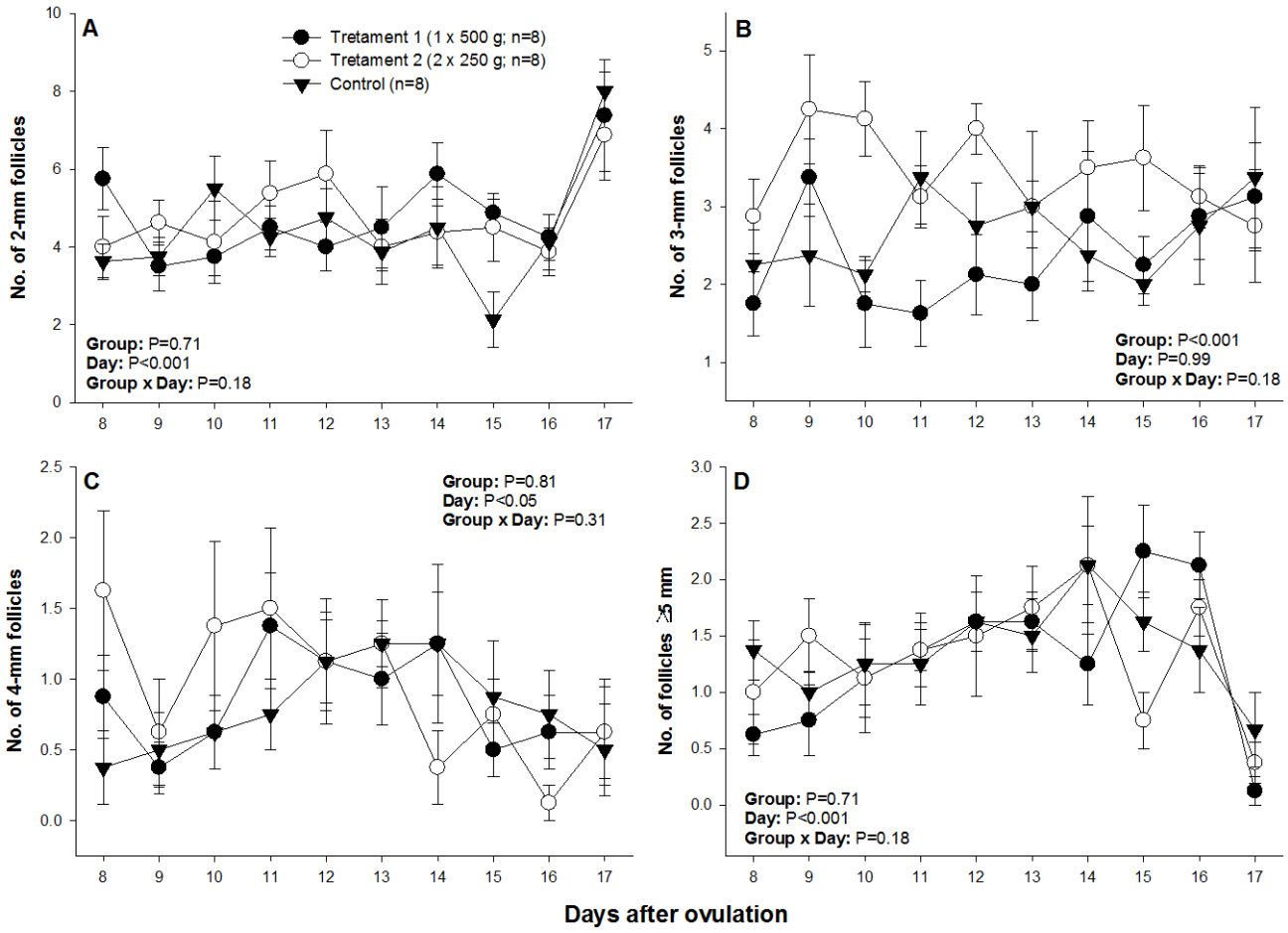

Fig. 1. Daily numbers (means±standrad SEMs of means) of ovarian antral follicles in different size classes recorded in ultrasonographically monitored cyclic Polish Mountain ewes that received lupin grain within 9-14 days after ovulation (Treatment 1: 1x500 g of lupin grain/day and Treatment 2: 2x250 g of lupin grain/day. See text for additional statistical details).

During the period from 1 day prior to lupin flushing to 3 days after, there was a significant main effect of day for the number of 2-, 4-, and ≥ 5-mm follicles, and a significant effect of group for the number of 3-mm follicles detected ultrasonographically in Polish Mountain ewes (Figure 1). In Treatment 1 ewes, the mean number of 2-mm follicles were greater on day 17 ($P<0.05$), compared with days 9 and 10. In Treatment 2 group, the number of 2-mm follicles was greater on day 17 ($P<0.05$) than on days 8, 9, 10, and 13-16. In addition, the mean number of 2-mm follicles on day 15 was higher in the Treatment 1 ewes than that in the Control sheep (Figure 1A). The mean daily numbers of 3-mm follicles were overall greater in Treatment 2 ewes (3.4±0.2 follicles/day; $P<0.05$), compared with those in Treatment 1 and Control animals (2.4±0.2 and 2.6±0.2 follicles/day, respectively). The difference between Treatment 2 and the other two groups was most prominent on day 10 (Figure 1B). Even though the main effect of day was significant for daily numbers of 4-mm follicles, subsequent statistical tests did not reveal any differences among the three subsets of

animals on any day of the observation period (Figure 1C). Lastly, the mean daily numbers of large antral follicles (≥5 mm in diameter) were lower on day 17 ($P<0.05$), compared with those on days 12, 13, 15, and 16. Moreover, they were lower on days 8 and 9 ($P<0.05$) than on days 15 and 16 in Treatment 1 ewes, whereas in the remaining two groups, the only significant difference was observed between days 17 and 13 (Control ewes) or between days 17 and 14 (Treatment 2 ewes; Figure 1D).

Discussion

Generally, nutritional flushing with lupins boosts fertility in non-prolific genotypes of sheep by increasing the incidence of twinning (Hill, 1988; Scaramuzzi et al., 2011). Significant increases in ovulation rates after the flushing are mainly observed in ewes receiving poor quality feed (e.g., animals grazing wheat straw or dry subterranean clover) (Hill, 1988). It has been found out that older animals usually respond better to nutritional flushing than younger ewes; however, in some studies, the age of ewes did not appear to be a determining factor (Hill, 1988). The results of our study showed that despite some changes in antral follicle numbers and wave dynamics, lupin grain feeding from 9 to14 days after ovulation did not consistently stimulate more ovulations in sexually mature, non-prolific ewes.

Failure to achieve a flushing response could occur because the Polish Mountain ewes had already reached their full genetic potential for ovulation rate with their previous nutritional regimen (the static effect of nutrition) (Coop, 2009). Alternatively, the inability to maintain the threshold level for digestible energy intake might explain lower than expected ovulatory responses (Coop, 2009). In the past, the amount of lupin used to improve sheep fertility varied considerably (from 170 to 750 g/day). To the best of our knowledge, there has been no systematic study of the effects of daily lupin grain doses in different breeds of sheep.

Our observations also indicate that the frequency of lupin feeding may affect ovarian responses in cyclic ewes.It is feasible that a series of twice daily or even more frequent lupin supplementations may be necessary to attain the required metabolic threshold and elicit ovarian stimulation. The administration of 500 g of lupin grain once a day (Treatment 1) did not increase the daily number of antral follicles. It merely shortened the regression phase of follicle lifespan (penultimate wave of the estrous cycle) by ~1.5 days, without apparent effects on other aspects of follicle wave development (e.g., interwave interval and maximum follicle diameter). On the other hand, feeding two meals of 250 g of lupin grain per day (Treatment 2), in the morning and evening, was associated with increased small (3-mm) follicle numbers, the prolonged growth phase of all emerging follicles, and greater size of ovulatory follicles emerging in the penultimate wave before ovulation, and earlier emergence of the last wave of the estrous cycle studied. Although there was a 3.4-fold increase in the percentage of ovulatory antral follicles emerging in the penultimate wave of the cycle in Treatment 2 ewes (Table 1), this difference was only numerical; such follicles were recorded in one control and two Treatment 2 animals. Our results contrast with those of the previous studies indicating the increased number of large and medium-sized follicles in response to short-term lupin with feeding (the latter was approximately doubled in response to lupin grain supplementation (Muñoz-Gutiérrez et al., 2002; Muñoz-Gutiérrez et al., 2005). Furthermore, our findings do not support an earlier suggestion made by Scaramuzzi et al. (2011) that the stimulating effects of supplemental nutrition late in the luteal phase on the final follicular wave are likely to be more pronounced than those exerted on the preceding waves of the interovulatory period in ewes. In the present study, short-term flushing using lupin grain started mid-way through the interovulatory interval, appeared to have a limited effect on the development of ovulatory follicles

emerging just before the onset of behavioral estrus. In fact, the proportion of ovulating antral follicles that emerged in the last wave of the cycle decreased numerically (by 21.7% and 27.9% in Treatment 1 and Treatment 2 ewes, respectively; Table 1). This can be explained by the fact that in the Control group, only 2/8 ewes ovulated ≤ 50% of all ovulatory sized follicles in the final wave, while 4/8 and 6/8 animals in the Treatment 1 and Treatment 2 groups had ≤ 50% ovulatory follicles in the final wave of the cycle, respectively.

In prolific breeds of sheep, a proportion (i.e., 50-75%) of large antral follicles in the penultimate wave of the estrous cycle, along with those emerging in the final follicular wave before estrus, are maintained, whereas ovulation of follicles from the second last wave in non-prolific ewes are sporadic (≤10%) (Bartlewski et al., 1999). Ovulatory follicles from the penultimate wave emerge ~48 h later than anovulatory follicles in the same wave. Therefore, their regression may be prevented by the next increase in serum concentrations of follicle-stimulating hormone (FSH) and/or by gradual increase in luteinizing hormone pulsatility at luteolysis. In addition, approximately 25% of all ovulatory sized follicles emerging in the last follicular wave in non-prolific ewes become atretic and do not ovulate, as opposed to near 100% ovulation of such follicles in this wave in prolific ewes (Bartlewski et al., 1999). All these aspects of antral follicular kinetics, except for the number of follicles ovulating in the last follicular wave, are due to the interbreed differences in circulating progesterone (P_4) concentrations during the luteal phase of the estrous cycle governing the timing of FSH peaks and initial stages of wave development. The number and percentage of ovulating follicles emerging in the last wave of the cycle appear to be independent of P_4 and FSH secretion (Baby and Bartlewski, 2011a; Baby and Bartlewski, 2011b; Bartlewski et al., 2017). By comparing those earlier observations with our results, it is doubtful that lower than normal luteal phase levels of P_4, seen in small ruminants receiving high-energy, high-protein diets (Hashem, 2012) were responsible for the alterations in wave dynamics in the ewes of the present study. These effects seem to be related mainly to high serum concentrations of glucose (Leury et al., 1990; Scaramuzzi et al., 2011) and insulin (Scaramuzzi et al., 2011) during the pre-ovulatory period, especially in animals receiving lupin grain twice a day. Elevated insulin levels associated with lupin grain flushing in the ewes increase ovarian glucose entry rates and stimulate FSH secretion and an increase in the number of large antral follicles (Scaramuzzi et al., 2011). The effect of nutrition on FSH concentrations are, however, very rapid and transient (Scaramuzzi et al., 2011). Follicle-stimulating hormone is responsible for the early stages of antral follicle development up to the attainment of their maximum diameter (Bartlewski et al., 2011). It is not known why lupin-induced changes in FSH secretion and/or bioavailability potentially resulting in prolonged follicular growth in the ewes of the present study, would be restricted to animals fed lupins twice a day.

In summary, feeding a total amount of 500 g of lupin grain per day, from days 9 to 14 after ovulation in estrus-synchronized ewes, had no effect on the ensuing ovulation rate. It was found out that lupin grain supplementation once a day had little influence on antral follicular development (monitored with transrectal ultrasonography). However, supplementing 250 g of lupin twice a day altered the duration of the follicular growth phase (penultimate wave) and advanced the emergence of the last wave of the estrous cycle, yet still failed to increase the number of ovulatory follicles. The present observations on antral follicle kinetics suggest that the effects of lupin grain on antral follicle growth are rapid and transient. Furthermore, they do not seem to be governed by progesterone-mediated alterations in circulating FSH concentrations documented in earlier experimental studies using ultrasonographically monitored ewes. More studies should be performed to confirm a hormonal basis for ovarian effects of nutritional flushing in small ruminants.

Authors' Contribution

Study concept and design: M. M., T. S., R. T. K., and P. M. B.
Acquisition of data: M. M., T. S., V. E., J. S., and P. M. B.
Analysis and interpretation of data: V. E., B. A., R. T. K., and P. M. B
Drafting of the manuscript: V. E. and P. M. B.
Critical revision of the manuscript for important intellectual content: M. M., T. S., R. T. K., and P. M. B.
Statistical analysis: B. A. and P. M. B.
Administrative, technical, and material support: M. M., T. S., and P. M. B.
Study supervision: M. M., T. S., and P. M. B.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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